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## Cloning of the Genes for AF/R1 Pili from Rabbit Enteroadherent *Escherichia coli* RDEC-1 and DNA Sequence of the Major Structural Subunit

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**AF/R1 pili on the surface of *Escherichia coli* RDEC-1 promote attachment of the bacteria to rabbit intestinal brush borders. In order to characterize AF/R1 pili and manipulate their expression, we cloned the genes necessary for AF/R1 expression; determined the size of proteins produced in minicells; located the gene encoding the major structural subunit, named *AfrA*; and determined the DNA sequence of *afrA* as well as the sequence of 700 additional nucleotides upstream of *afrA*. Two contiguous *EcoRI* fragments spanning 7.9 kilobases were cloned from the 86-megadalton plasmid of RDEC-1 into vector pUC19 to make plasmid pW1. Bacteria carrying pW1 produced AF/R1 pili that were recognized by AF/R1-specific antiserum and promoted adherence of bacteria to brush borders prepared from rabbit intestine. Proteins with a molecular weight of 17,000 (17K proteins), which was the size of *AfrA*, as well as 15K, 15.5K, 26K, 28K, and 80K proteins were detected in minicells carrying pW1. The gene *afrA* was located by using an oligonucleotide probe, and its DNA sequence was determined. The DNA sequence of 700 additional nucleotides upstream was determined because this sequence may be important in the regulation of AF/R1 expression.**

AF/R1 pili are surface antigens on *Escherichia coli* RDEC-1 that promote adherence of the bacteria to rabbit intestine. Mutants of RDEC-1 that do not express AF/R1 are virulent, but disease is less severe than that caused by the wild-type strain (7, 27). AF/R1 pili promote adherence to brush borders prepared from the intestines of rabbits but not from those of other species (10) and are responsible for the initial adherence of RDEC-1 bacteria to epithelial cells in the gut, providing access to the surface of the epithelial cells, where unidentified bacterial virulence factors then efface the microvilli (6).

AF/R1 antigens are typical pilus structures on the bacterial surface (5; F. J. Cassels et al., manuscript in preparation). The AF/R1 pilus is composed of protein subunits, and the major subunit has an apparent molecular weight of 19,000 (5). The amino acid sequence of the N terminus of this major subunit has been determined and has features in common with type 1, Pap, and K99 pili (Cassels et al., in preparation). Expression of AF/R1 pili is mediated by an 86-megadalton (MDa) plasmid (9), and a 2.4-kilobase (kb) *EcoRI* fragment from the 86-MDa plasmid has been shown to be important since three separate *Tn5* insertions in this fragment resulted in the loss of AF/R1 expression (27).

We cloned the genes that are necessary for AF/R1 expression in a high-copy-number plasmid, mapped the cloned DNA, characterized the proteins encoded by the clone, and obtained the DNA sequence of the major subunit.

Fragments from the 86-MDa plasmid that mediates expression of AF/R1 pili were obtained after partial digestion of plasmid DNA with *EcoRI* and ligated to pUC19 (20, 27). Transformants of *E. coli* DH5 $\alpha$  (purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md.) were screened by using antiserum specific for AF/R1 pili (27, 28), and an AF/R1 clone was obtained. The anti-AF/R1 antise-

rum recognized both the intact pilus structure and the major subunit that was obtained after dissociation of pili, so that transformants expressing either the intact pilus structure or the subunit on the surface would be detected.

The plasmid in this clone was designated pW1 and was composed of pUC19 plus an insert of 2.4- and 5.5-kb *EcoRI* fragments from the 86-MDa plasmid. AF/R1 antigen expression by DH5 $\alpha$  carrying pW1 was confirmed by slide agglutination, using antiserum specific for AF/R1. A map of pW1 is given in Fig. 1. Only these contiguous 2.4- and 5.5-kb *EcoRI* fragments were necessary for expression of the AF/R1 antigen that functioned in adherence. This was somewhat unexpected, since a deletion derivative of the 86-MDa plasmid that retains these fragments does not express AF/R1 (27). It seems likely that the high copy number of pW1 may supplant a requirement of the native large plasmid.

Plasmid pW1 mediated expression of the AF/R1 antigen on the surface of both strains DH5 $\alpha$  and HB101 (Table 1). The AF/R1 pili encoded by pW1 were shown to function in adherence, since they promoted attachment of the bacteria to intestinal brush borders isolated from rabbits (8). Significant numbers of DH5 $\alpha$  isolates carrying pW1 adhered to rabbit brush borders (an average of  $4.7 \pm 2.8$  bacteria per brush border) compared with the numbers of DH5 $\alpha$  isolates carrying the vector pUC19 that adhered to rabbit brush borders (an average of  $0.3 \pm 2.3$  bacteria per brush border). Binding mediated by plasmid pW1 in DH5 $\alpha$  was substantially less than that mediated by RDEC-1 grown in Penassay broth (PAB; Difco Laboratories, Detroit, Mich.) (greater than 10 bacteria per brush border).

Expression of AF/R1 from the native 86-MDa plasmid is inhibited by growth in brain heart infusion medium (BHI) and low growth temperature (9), so expression from pW1 was tested under these conditions. Strains DH5 $\alpha$  and HB101 carrying pW1 expressed AF/R1 antigen whether they were grown in PAB or BHI (Table 1), but strains RDEC-1 and M5

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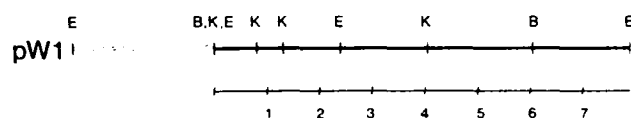


FIG. 1. Map of plasmid pW1. The arrow indicates the direction of transcription from the *lac* promoter of pUC19. Abbreviations: E, *EcoRI*; B, *BamHI*; K, *KpnI*. The scale at the bottom indicates kilobase pairs of cloned DNA.

(an HB101 derivative), which carry the entire 86-MDa plasmid, did not express AF/R1 when grown in BHI. Likewise, growth of bacteria in BHI did not affect adherence of DH5 $\alpha$  carrying pW1 to brush borders ( $3.0 \pm 2.3$  adherent bacteria per brush border) but reduced adherence of RDEC-1 to  $0.2 \pm 0.5$  bacteria per brush border. This indicates that functional AF/R1 pili were expressed from pW1, even when the bacteria were grown in BHI.

When the 86-MDa plasmid from strain M5 was conjugated into DH5 $\alpha$  carrying pW1, normal regulation by the growth medium was restored (Table 1). This suggests that a repressor is produced by the 86-MDa plasmid that acts *in trans* to repress AF/R1 expression by pW1. This repressor is not expressed by the DNA cloned on pW1, and the gene may not be included in the clone.

In contrast, expression of AF/R1 was suppressed by growth at a low temperature whether it was encoded by pW1 or by the 86-MDa plasmid (Table 1). This temperature effect was specific for the AF/R1 genes and was not a feature of the *lac* promoter from the vector since bacteria carrying pUC19 expressed the Lac<sup>+</sup> phenotype whether they were grown at 37 or 22°C (data not shown). It is not known whether the regulation is at the level of transcription, translation, or synthesis and assembly. All known *E. coli* pilus systems except type 1 are similarly controlled by growth temperature (18, 25). The mechanism of the control is largely unknown, but in those pilus systems that have been studied, temperature was shown to regulate transcription (14, 22, 26), so it seems likely that the cloned DNA includes sequences that can respond to transcriptional control.

Minicells were prepared from *E. coli* DS410 carrying plasmids pW1 and pUC19 (23), and plasmid-encoded proteins were labeled with [<sup>35</sup>S]methionine. A prominent protein with a molecular weight of 17,000 was expressed in minicells carrying pW1 but not in minicells carrying pUC19



FIG. 2. Minicell analysis of proteins expressed by pW1. Proteins in minicells carrying pW1 or pUC19 were labeled with [<sup>35</sup>S]methionine, separated by polyacrylamide gel electrophoresis, and detected by autoradiography. Proteins were labeled with [<sup>35</sup>S]methionine (1.151 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose (28), and detected by autoradiography.

(Fig. 2). The mass of the AF/R1 pilus subunit has been reported as 19 kDa (5), but in this gel system it ran at 17 kDa. Two smaller proteins of approximately 15.5 and 15 kDa and proteins of 26, 28, and 80 kDa were also unique to pW1. Both pW1 and pUC19 encoded proteins of 30 and 32 kDa. The appearance of proteins other than the major subunit was consistent with other pilus systems which require genes for synthesis and assembly of pili and have minor subunits that are important for adherence (1, 11, 14–16, 21), proper length (3, 21), and anchorage to the bacterial surface (3). The larger proteins seen in this study may be important for synthesis and assembly, and the smaller proteins may be minor subunits analogous to those found in other pili.

The location of the structural gene for the AF/R1 subunit was mapped by using an oligonucleotide probe. The oligonucleotide was an equimolar mixture with the sequences CAG GGT/C GAT/C GTT/A/G CAG TTC TTC GG corresponding to the amino acid sequence Gln Gly Asp Val Gln Phe Phe Gly of the N-terminal sequence of the major subunit (Cassels et al., in preparation). It hybridized (20, 24) to the 2.4-kb *EcoRI* fragment of the 86-MDa plasmid from M5, to this fragment in clones, and to a subclone carrying only the *EcoRI*-*KpnI* fragment to the far left of the cloned DNA (data not shown). It did not hybridize to other fragments. These data indicate that DNA encoding the N-terminal portion of the AF/R1 structural subunit is within the 700-base-pair *EcoRI*-*KpnI* fragment of cloned DNA. This fragment is within the first kilobase of cloned DNA, as drawn in Fig. 1.

The DNA sequence of 1,126 bases of this *EcoRI*-*KpnI* fragment and the neighboring *KpnI*-*KpnI* fragment was obtained and is given in Fig. 3. The mature AF/R1 major structural subunit was encoded beginning at base 690. This was identified by comparison with the N-terminal amino acid sequence (Cassels et al., in preparation). The protein ended at a stop codon at base 1113. A leader sequence for the protein was elucidated as having two methionine codons that could encode the first amino acid. The first amino acid of the protein is most likely encoded beginning at base 627, because there is a sequence 10 base pairs upstream from base 627 that matches the consensus sequence for a ribosome-binding site (19). The precursor protein has 162 amino acid residues and a calculated molecular mass of 16,562 Da. The mature protein has 141 amino acid residues and a calculated

TABLE 1. Expression of AF/R1 pili in transformants

Strain	Expression under the following growth conditions <sup>a</sup> :		
	PAB, 37°C	BHI, 37°C	PAB, 22°C
DH5 $\alpha$ (pW1)	+3	+3	0
DH5 $\alpha$ (pUC19)	0	0	0
HB101(pW1)	+4	+2	0
HB101(pUC19)	0	0	0
M5	+4	0	0
RDEC-1	+4	0	0
DH5 $\alpha$ (pW1, pM5)	+3	0	0

<sup>a</sup> Expression was determined by slide agglutination with AF/R1-specific antiserum. The reactions were scored from 0 to +4 as described previously (27).

1	GAATTCCTTA GTGAATGTCT GCTGGGAATC ATAAAACAAT CTTTCTGATA
51	TATCCACAAT TTTTAGGTTG GTAAATCTTA AAAGAATAGC CGCTCGCGTT
101	ATCCTGCTTA ATTGAATGTA TTTACCTAAA GTAACACCTA TGTTTTCTTT
151	AAACAGTAAT TGCAGATACC GTCTGCTGTA TCCGGAGTAA TCAACGAGGG
201	CATTTATATC TATAGATATA CTCTCTAAAT TATCATCAAT GACTGTGT
251	ATCGCGTTTA TCGTAAGTGT TTTCAGCATG TACGTAGCTC CTATATGTAT
301	GTTTACGTGT TACCCACAT CATGTTAATA AAACCCCTTC TGTTTTTTTA
351	<u>GCTGATTGTG</u> <u>CATTGTACAC</u> <u>ATACCGTGCA</u> <u>CAATTAGCTA</u> ACAACGCAGA
401	CCAATATTTT TTAAATACC CCGCGTTTTC ACATGACTTG TATCTATTCT
451	CTTAGAGAAA <u>TTAATGCATC</u> <u>TCTATCACAT</u> <u>CATGTGTAGT</u> <u>ACTGGACAAA</u>
501	TAGTCATGGG AGCCTATTAC <u>CGAACAGCGA</u> AGATGGCATA TGTTTTCTTA
551	TTAAGAAAGA GGAAAGAATA TGGCGCACTC GTTTTATGTC AATTTTGTA
601	AAAAAATATA <u>TGGAG</u> AATGTCAGAA ATG AAA AAA ACA TTT ATT GCG
648	Ser Val Ile Val Ile Thr Ile Asn Thr Gly Ser Ala Ile Ala TCT GTA ATT GTA ATA ACC ATA AAT ACG GGA TCA GCA ATT GCT
690	Ala Gln Gly Asp Val Gln Phe Phe Gly Thr Val Thr Ala Lys GCT CAA GGC GAT GTT CAG TTC TTT GGT ACC GTT ACT GCC AAG
732	Thr Cys Asp Leu Val Val Glu His Glu Gly Ala Val Val Asn ACC TGT GAT CTT GTC GTT GAA CAC GAG GGG GCT GTG GTC AAT
774	Met Ile Gln Leu Gly Ser Val Thr Asn Gly Gly Thr Asn Ala ATG ATT CAG TTG GGT TCT GTA ACT AAT GGT GGA ACT AAT GCT
815	Gly Thr Asp Ile Gly Ala Asn Lys Ser Phe Thr Leu Lys Pro GGC ACC GAT ATC GGA GCA AAC AAA TCG TTT ACC CTG AAG CCA
858	Ala Ser Gly Val Thr Cys Asn Thr Ile Thr Thr Ala Lys Met GCA TCA GGG GTG ACA TGC AAT ACC ATC ACT ACT GCT AAA ATG
900	Ala Trp Ser Ser Pro Ala Met Thr Val Asn Gly Ile Gly Asn GCA TGG TCT TCT CCT GCA ATG ACC GTT AAT GGT ATT GGT AAT
942	Leu Ser Gly Lys Ala Ile Asp Ala His Val Lys Leu Val Ala CTA TCA GGT AAG GCT ATT GAT GCC CAT GTG AAG TTA GTG GCG
984	Ile Asn Ser Thr Gly Lys Val Gln Thr Asp Thr Asn Ala Asp ATT AAC AGC ACG GGT AAA GTT CAA ACT GAT ACC AAC GCA GAT
1026	Lys Glu Ile Lys Ala Gly Gln Asn Thr Val Asp Tyr Ser Ile AAG GAA ATT AAA GCG GGT CAA AAT ACA GTT GAT TAC TCA ATT
1068	Thr Gly Ser Gly Leu Leu Met Lys Ala Leu Asn Leu Lys Leu ACT GGT TCT GGC CTA CTG ATG AAG GCT TTA AAT TTA AAG CTC
1110	Ser *** AGT TAA TTGGCGGTACC

FIG. 3. DNA sequence of the left part of the cloned DNA. The deduced amino acid sequence of AfrA, the major structural subunit of AF/R1, is given. -35 and -10 indicate putative promoters that matched consensus sequences for binding of RNA polymerase to initiate transcription. RBS indicates DNA that matched the consensus sequence for ribosome-binding sites to initiate translation. Underlined segments of DNA indicate an inverted repeat that may promote a stem-loop structure. Underlined and overlined segments of DNA indicate sequences homologous to the regulatory region of Pap DNA. The three asterisks indicate a stop codon for translation into amino acids. DNA was sequenced by Pan-Data Systems, Inc. (Rockville, Md.) by the dideoxy method. Analysis was performed by using software developed by the University of Wisconsin Genetics Computer Group (12).

molecular mass of 14,401 Da. We designated this gene *afrA* as the major structural gene for the AF/R1 pilus. The deduced amino acid sequence is given in Fig. 3.

The amino acid sequence of the AF/R1 major structural gene revealed that it belongs to a group of pili related to type 1 pili, as described by Klemm (18), in which every other amino acid in a region near the N terminus is conserved (this report; Cassels et al., in preparation). The DNA sequence of the entire *afrA* gene was 43% homologous to the DNA encoding the FimA subunit of the type 1 pilus (17) and 42% homologous to the sequence encoding the PapA pilus (4). The 42 nucleotides in the region encoding the conserved amino acids were, likewise, 43% homologous to the DNA encoding similar areas of FimA and PapA, so were no more conserved than the rest of the protein was.

Although the cloned DNA had little homology with the *pap* pilus operon, 20 of 30 bases beginning at base 461 (including 11 of 13 bases beginning at base 475) matched a sequence from the *pap* pilus operon that has been implicated as a binding site for the catabolite repressor protein that is involved in catabolite repression (2). The match for the consensus sequence for the catabolite repressor protein-binding site described by Ebright et al. (13), however, was poor. Furthermore, there is no evidence that AF/R1 expression responds to catabolite repression (W. H. Cohen, M. S. thesis, The Catholic University of America, Washington, D.C., 1986). The significance of the homology with the *pap* DNA is unknown, but we speculate that this region may serve as a DNA-binding site for some regulatory protein (perhaps for temperature or growth medium regulation).

The DNA upstream of *afrA* contained one open reading frame beginning at base 279 and extending through the sequenced DNA in the opposite orientation from that encoding the AF/R1 major structural subunit. Because its position is analogous to that of PapI, a regulatory protein in the *pap* pilus operon (2), we suspect that it may be involved in expression of AF/R1 pili. Further work is necessary to define how the AF/R1 genes are regulated.

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